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Bescheinigung | Certificate | Attestation

Die angehefteten Unterlagen stimmen mit der ursprünglich eingereichten Fassung der auf dem nächsten Blatt bezeichneten europäischen Patentanmeldung überein.

The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

99306887.3

PRIORITY DOCUMENT

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
p.o.

I.L.C. HATTEN-HECKMAN

DEN HAAG, DEN
THE HAGUE,
LA HAYE, LE 11/10/00

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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

Anmeldung Nr.:
Application no.:
Demande n°: 99306887.3

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Anmelder:
Applicant(s):
Demandeur(s):
NYCOMED AMERSHAM PLC
Amersham Buckinghamshire, HP7 9LL
UNITED KINGDOM

Bezeichnung der Erfindung:
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Titre de l'invention:
Nucleoside analogues

In Anspruch genommene Priorität(en) / Priority(ies) claimed / Priorité(s) revendiquée(s)

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International Patent classification:
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C07H19/052, C07H21/00, C07D249/12, C07D249/14, C07D233/66

Am Anmeldetag benannte Vertragstaaten:
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Remarks:
Remarques:

NUCLEOSIDE ANALOGUES

Nucleic acids are manipulated *in vitro* in a wide variety of research and diagnostic techniques. The methods can involve the synthesis of nucleic acid probes by means of polymerase or terminal transferase enzymes for the purposes of labelling or determination of base sequence identity. Labelling often involves the incorporation of a nucleotide which is chemically labelled or which is of a particular chemical composition so as to make it detectable. Nucleic acid probes made in this way can be used to determine the presence of a nucleic acid target which has a complementary sequence by means of hybridisation of the probe to the target.

In WO 94/21658 T I Kalman describes novel nucleoside or nucleotide analogues having a 4-acetylimidazolin-2-one base and their use for inhibiting virally encoded reverse transcriptases.

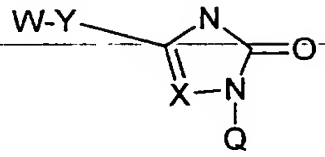
In Z Naturforsch B, 1986, 41b (12), 1571-9, T Fukuda *et al* describe the effect of incorporation of nucleoside analogues having an imidazolin-2-one base as both T and G in DNA duplexes.

In Tetrahedron Letters, 40 (1999), 835-838, E Bedu *et al* describe the preparation of a nucleoside analogue having a 4-amidoimidazolin-2-one base and used as a cytosine analogue in triple helix forming oligonucleotides.

Purine and pyrimidine base nucleosides and nucleotides have been derivatised with reporter groups and are well known and widely used for labelling DNA or RNA and in other molecular biology applications. But these molecules are often poor enzyme substrates. There is a continuing need for labelled nucleoside analogues whose triphosphates are good enzyme substrates.

According to the present invention there is provided a

compound having the structure



where X is CH or N,

Y is -CO-, -CONW-, -O-, -S-, -SO-, -SO_2-, -NWCO-,-NW-, or -OCO-,

W is the same or different at different places in the molecule and each is H or alkyl or aryl or Rp or –Ln–Rp,

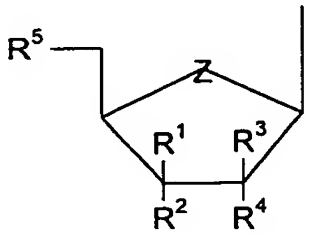
Ln is a linker group,

Rp is a reporter moiety, and

Q is H or a sugar or a sugar analogue or a nucleic acid backbone or backbone analogue,

provided that at least one reporter moiety Rp is present.

Q may be



where Z is O, S, Se, SO, NW or CH_2,

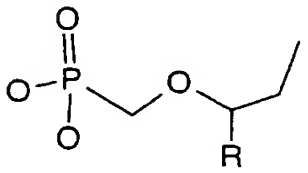
R^1, R^2, R^3 and R^4 are the same or different and each is H, OH, F, NH_2, N_3, O-hydrocarbyl or Rp or –Ln–Rp,

R^5 is OH, SH or NH_2 or mono-, di- or tri-phosphate or -thiophosphate, or corresponding boranophosphate,

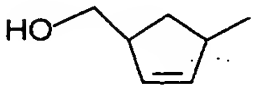
or one of R^2 and R^5 is a phosphoramidite or other group for incorporation in a polynucleotide chain, or Rp or –Ln–Rp,

or Q consists of one of the following modified sugar structures

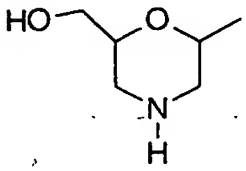
Acyclic Sugars

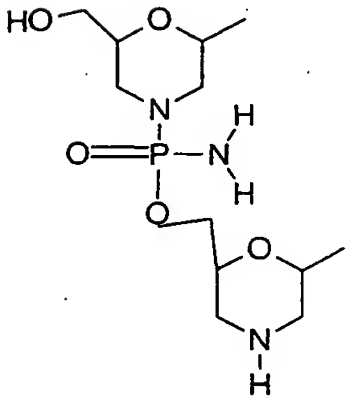


R= CH_3 , CH_2OH , H ,



Morpholino Backbone





or Q is a nucleic acid backbone consisting of sugar-phosphate repeats or modified sugar-phosphate repeats (e.g. LNA)

(Koshkin *et al*, 1998, Tetrahedron 54, 3607-30) or a backbone analogue such as peptide or polyamide nucleic acid (PNA). (Nielsen *et al*, 1991, Science 254, 1497 - 1500).

When Q is H, these compounds are base analogues. When Q is a sugar or sugar analogue or a modified sugar, these compounds are nucleotide analogues or nucleoside analogues. When Q is a nucleic acid backbone or a backbone analogue, these compounds are herein called nucleic acids or polynucleotides.

A nucleoside analogue is a molecule which is capable of being incorporated, either chemically or enzymatically, into an oligomeric or polymeric nucleic acid (DNA or RNA) chain, and when so incorporated of forming a base pair with a nucleotide residue in a complementary chain or base stacking in the appropriate nucleic acid chain.

In the context of this invention, a nucleotide is a naturally occurring compound comprising a heterocyclic base and a backbone including a phosphate. A nucleoside is a corresponding compound in which a backbone phosphate may or may not be present. Nucleotide analogues and nucleoside analogues are analogous compounds having different bases and/or different backbones. A nucleoside analogue is a compound which is capable of forming part of a nucleic acid (DNA or RNA or PNA) chain, and is there capable of base-pairing with a base in a complementary chain or base stacking in the appropriate nucleic acid chain. A nucleoside analogue may be specific, by pairing with only one complementary nucleotide; or degenerate, by base pairing with more than one of the natural bases, e.g. with pyrimidines (T/C) or purines (A/G); or universal, by pairing with each of the natural bases with little discrimination; or it may pair with another analogue or itself.

In one preferred aspect of the invention, the base analogue is linked to a sugar moiety such as ribose or deoxyribose to form a nucleoside analogue. When the group R^5 is triphosphate, the nucleoside triphosphate analogues of the invention are capable of being incorporated by enzymatic

means into nucleic acid chains.

A reporter moiety Rp may be any one of various things. It may be a radioisotope by means of which the nucleoside analogue is rendered easily detectable, for example 32-P or 33-P or 35-S incorporated in a phosphate or thiophosphate or phosphoramidite or H-phosphonate group, or alternatively 3-H or 14-C or 125-I. It may be a stable isotope or a specific chemical moiety suitable for detection by mass spectrometry. (Or the compound as a whole may be suitable for detection by mass spectrometry.) It may be a signal moiety e.g. an enzyme, hapten, fluorophore, chemiluminescent group, Raman label or electrochemical label.

The reporter moiety may be a solid surface, to which the nucleoside analogue is attached and by means of which it may be distinguished from nucleoside analogues not so immobilised. The reporter moiety may be a reactive group, either a nucleophilic group, e.g. NH_2, OH, COOH, $CONH_2$, ONH_2, SH or a thiophosphate or a hydrazine or a hydrazide, or an electrophilic group e.g. an active ester or aldehyde or maleimide, by which a signal moiety and/or a solid surface may be attached, before or after incorporation of the nucleoside analogue in a nucleic acid chain. Such reporter groups are well known and well described in the literature.

A linker group Ln is a chain of 1 to 60 or more carbon, nitrogen, oxygen phosphorus and/or sulphur atoms, rigid or flexible, saturated or unsaturated, as well known in the field. Preferably the linker group is joined to a 4-triazole ring (when X is N) or to a 4-imidazole ring (when X is CH) of the nucleoside analogue molecule by an amide or amine bond. Preferably the linker group is joined to the reporter moiety by an amide bond.

To avoid risk of steric hindrance, a linker preferably has at least three chain atoms, e.g. $-(CH_2)_n$ - where n is at least 3.

Two (or more) reporter moieties may be present, e.g. a signal

moiety and a solid surface, or a hapten and a different signal moiety, or two fluorescent signal groups to act as donor and acceptor. Various formats of these arrangements may be useful for separation or detection purposes.

Purine and pyrimidine nucleoside derivatives labelled with reporter moieties are well known and well described in the literature. Labelled nucleoside derivatives have the advantage of being readily detectable during sequencing or other molecular biology techniques.

R^1, R^2, R^3 and R^4 may each be H, OH, F, NH_2, N_3, O-alkyl or a reporter moiety. Thus ribonucleosides, and deoxyribonucleosides and dideoxyribonucleosides are envisaged together with other nucleoside analogues. These sugar substituents may contain a reporter moiety in place of or in addition to the one or two present in the base.

R^5 is OH or mono-, di- or tri-phosphate or -thiophosphate or corresponding boranophosphate. From nucleosides (R^5 is OH) it is readily possible to make the corresponding nucleotides (R^5 is triphosphate) by literature methods. Alternatively, one of R^2 and R^5 may be a phosphoramidite or H-phosphonate or methylphosphonate or phosphorothioate or amide, or an appropriate linkage to a solid surface e.g. hemisuccinate controlled pore glass, or other group for incorporation , generally by chemical means, in a polynucleotide chain. The use of phosphoramidites and related derivatives in synthesising oligonucleotides is well known and described in the literature.

In the new nucleoside analogues to which this invention is directed, at least one reporter moiety is present preferably in the base analogue and/or optionally in the sugar moiety or a phosphate group. Reporter moieties may be introduced into the sugar moiety of a nucleoside analogue by literature methods (e.g. J. Chem. Soc. Chem. Commun. 1990, 1547-8; J. Med. Chem., 1988, 31. 2040-8). Reporter moieties in the form of isotopic labels may be introduced into phosphate groups by literature methods (Analytical Biochemistry, 214, 338-340, 1993; WO 95/15395).

When R^5 is triphosphate, the nucleoside analogues are

available for enzymatic incorporation in DNA or RNA. The invention includes in another aspect the polynucleotide chain comprising at least one residue of the nucleoside analogue as defined.

Nucleoside analogues of this invention are useful for labelling DNA or RNA or for incorporating in oligonucleotides or PNA. A reporter moiety is attached at a position where it does not have a significant detrimental effect on the physical or biochemical properties of the nucleoside analogue, in particular its ability to be incorporated in single stranded or double stranded nucleic acid.

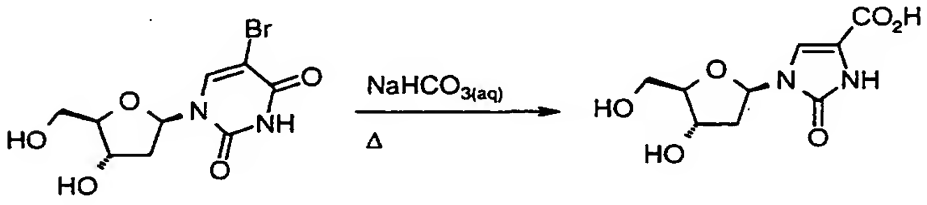
A template containing the incorporated nucleoside analogue of this invention may be suitable for copying in nucleic acid synthesis. If a reporter moiety of the incorporated nucleoside analogue consists of a linker group, then a signal moiety can be introduced into the incorporated nucleoside analogue by being attached through a terminal or other reactive group of the linker group.

A nucleoside analogue triphosphate of this invention may be incorporated by enzymes such as terminal transferase to extend the 3' end of nucleic acid chains in a non-template directed manner. Tails of the nucleoside analogue triphosphate produced in this way may be detected directly in the absence of any reporter label by use of antibodies directed against the nucleoside analogue. The analogues when incorporated into oligonucleotides or nucleic acids may be acted upon by nucleic acid modification enzymes such as ligases or restriction endonucleases.

The nucleoside analogues of this invention can also be used in any of the existing applications which use native nucleic acid probes labelled with haptens, fluorophores or other reporter groups, for example on Southern blots, dot blots and in polyacrylamide or agarose gel based methods or solution hybridisation assays and other assays in microtitre plates or tubes or assays of oligonucleotides or nucleic acids such as on microchips. The probes may be detected with antibodies targeted either against haptens which are attached to the base analogues or against the

base analogues themselves which would be advantageous in avoiding additional chemical modification. Antibodies used in this way are normally labelled with a detectable group such as a fluorophore or an enzyme. Fluorescent detection may also be used if the base analogue itself is fluorescent or if there is a fluorophore attached to the nucleoside analogue.

EXPERIMENTAL



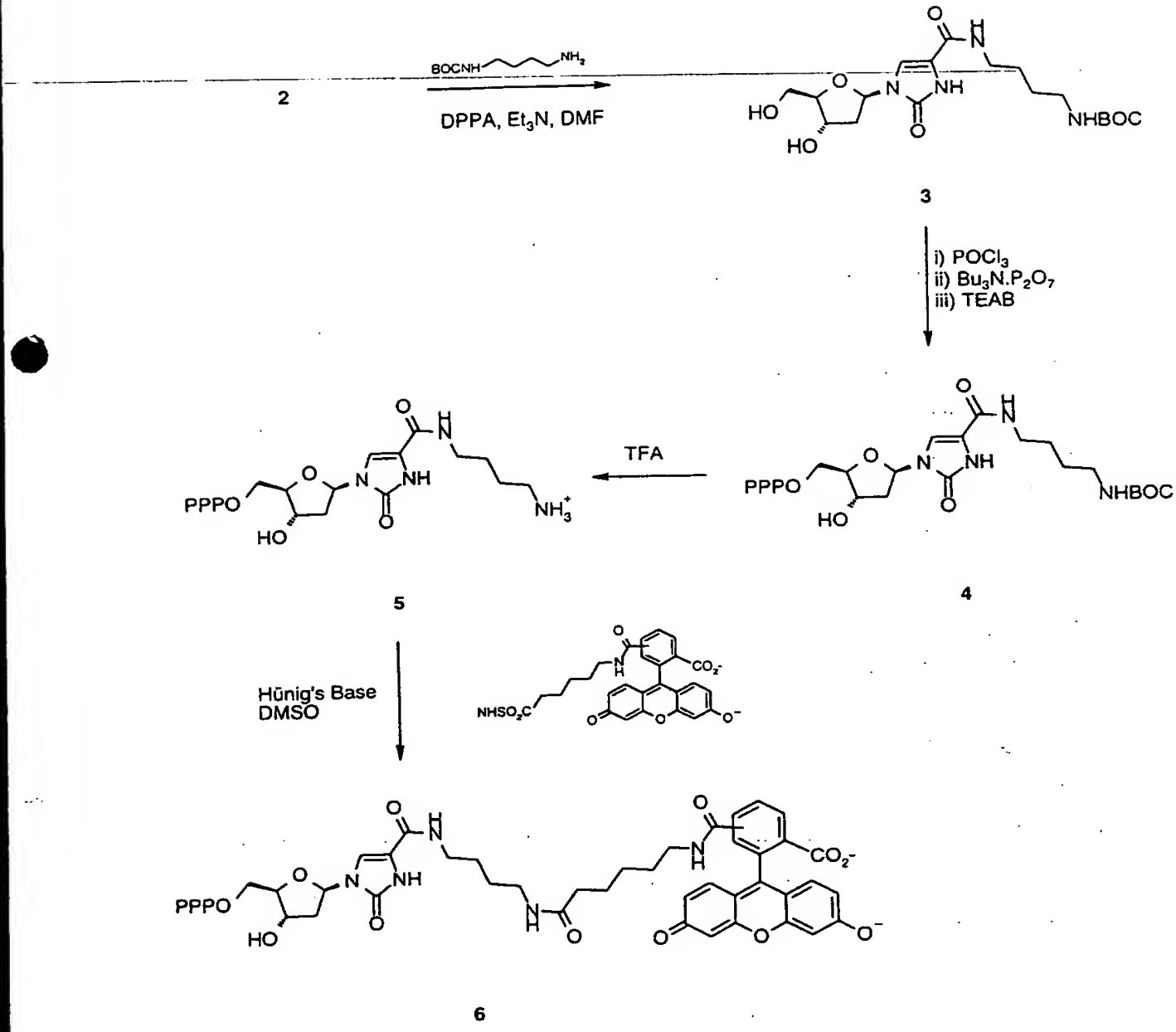
1 2

Example 1

Preparation of 1-(2'-deoxyribos-1'-yl)imidazolidin-2-one-4-carboxylic acid (2)

This was carried out according to the method of Otter *et al.* (J Org Chem, 1969, **34**, 1390). The acid was purified by reversed phase HPLC.

^{1}H nmr (D_2O) 2.20 (1H, m, 2'-CH_aH_b), 2.34 (1H, m, 2'-CH_aH_b), 3.50 (2H, m, 5'-CH_2OH), 3.86 (1H, m, 4'-CH), 4.33 (1H, m, 3'-CH), 5.87 (1H, t, J = 7 Hz, 1'-CH), 6.97 (1H, s, Ar-H).


2
BOCNH
NH2
DPPA, Et3N, DMF
HO
HO
NHBOC
3
i) POCl3
ii) Bu3N.P2O7
iii) TEAB
PPPO
HO
NHBOC
4
TFA
PPPO
HO
NH3+
5
Hünig's Base
DMSO
NHSO2C
CO2-
O-
PPPO
HO
CO2-
O-
6

Preparation of 4''-(N'-*tert*-butoxycarbonylamino)butyl 1-(2'-deoxyribos-1'-yl)imidazolidin-2-one-4-carboxamide (3)

The carboxylic acid (30 mg, 0.12 mmol) and 4-(N-*tert*-butoxycarbonylamino)-1-aminobutane (25 mg, 0.14 mmol) in dry DMF (1.5 ml) under a nitrogen atmosphere were treated with a solution of diphenylphosphoryl azide (39 mg, 0.14 mmol) in dry DMF (0.5 ml) and then dry triethylamine (0.04 ml). The mixture was allowed to stir at room temperature for 60 hours. The solvent was removed *in vacuo* to give a pale yellow solid that was purified by preparative tlc (RP18, 1:1 ethanol:water) then reversed phase HPLC to give 18.6 mg of the desired amide as a colourless oil.

^{1}H nmr (CD3OD) 1.42 (9H, s, C(C$\underline{H}_3$)), 1.55 (4H, m, BOCNHCH$_2$C$\underline{H}_2$C$\underline{H}_2$), 2.23 (1H, m, 2'-CH$_a\underline{H}_b$), 3.05 (2H, t, J = 6.6 Hz, BOCNHC$\underline{H}_2$), 3.30 (2H, obscured t, C$\underline{H}_2$NHC(=O)), 3.69 (2H, m, 5'-C$\underline{H}_2$OH), 3.88 (1H, m, 4'-C$\underline{H}$), 4.38 (1H, m, 3'-C$\underline{H}$), 5.99 (1H, t, J = 6.4 Hz, 1'-C$\underline{H}$), and 7.32 (1H, s, Ar-$\underline{H}$).

Preparation of 4''-(N'-*tert*-butoxycarbonylamino)butyl 1-(2'-deoxyribos-1'yl)imidazolidin-2-one-4-carboxamide-5'-triphosphate (4)

The nucleoside (**3**) (18.4 mg, 0.04 mmol) was dissolved in a 1:1 mixture of trimethylphosphate and triethylphosphate (2 ml) under an atmosphere of argon. The mixture was cooled to 0°C with an ice bath and phosphoroyl chloride (17 µl) was added dropwise and the mixture was stirred at 0°C for 2 hours. Tributylammonium pyrophosphate (0.44 ml of a 0.5M solution in dry DMF) was added, followed immediately by addition of tributylamine (50 µl). The mixture was stirred at room temperature for 15 minutes and the reaction was quenched by addition of 1M triethylammonium bicarbonate (5 ml). The mixture was stirred for 1 hour and then the solvents were removed *in vacuo*. The mixture was purified by ion exchange chromatography and then reversed phase chromatography to give a colourless solid. λ_{max} (H_2O) 264 nm, ^{1}Hnmr and ^{31}Pnmr (D_2O) were

consistent with the desired material, but showed that the compound was contaminated with triethylammonium pyrophosphate.

Preparation of 4''-aminobutyl 1-(2'-deoxyribos-1'-yl)imidazolin-2-one-4-carboxamide-5'-triphosphate trifluoroacetate salt (5)

The *tert*-butoxycarbonyl protected amine (6.6 µmol) was treated with trifluoroacetic acid (1 ml) at room temperature for 1.5 hours. The solvent was removed *in vacuo* to give the ammonium salt as a colourless solid. ^{1}H nmr showed the absence of the 9 proton singlet for the *tert*-butoxycarbonyl group and was otherwise consistent with the desired structure.

Preparation of 4''-(6'''-(fluorescein-5''''-(and 6'''')-carboxamidohexanamido)butyl 1-deoxyribos-1'-yl)imidazolin-2-one-4-carboxamide-5'-triphosphate (6)

The amine salt (**5**) was dissolved in anhydrous DMSO and treated with N,N-diisopropyethylamine (5 µl) and 6-(fluorescein-5-(and 6-) carboxamidohexanoic acid NHS ester (3.6 mg). The mixture was allowed to stir for 20 hours and the mixture was purified by ion-exchange chromatography . λ_{max} 486 nm, ^{1}H nmr was consistent with expected structure.

Example 2

A primer extension assay was used to evaluate compounds **(4, 5 and 6)** as a substrate for exonuclease free Klenow fragment DNA polymerase I (EFK). The assay used a ^{33}P 5' end labelled 15mer primer hybridised to a 24mer template. The sequences of the primer and template are:

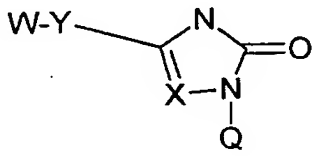
Primer	5' TGCATGTGCTGGAGA 3'
Template 1	3' ACGTACACGACCTCTGAACTAGTC 5'
Template 2	3' ACGTACACGACCTCTTGGCTAGTC 5'

One picomole ^{33}P labelled primer was hybridised to 2 picomoles of template in x2 Klenow buffer. To this was added either 4 µM dNTPαS or 40µM **(4 or 5)**, 20µM **(6)** or 160µM **(4 or 5)** or a mixture of 4 µM dNTPαS and 40µM **(4 or 5)** or 160µM **(4 or 5)**. One unit EFK and 2 mU **(4 or 5)** or 20 mU **(6)** inorganic pyrophosphatase were used per reaction. Primer alone, primer plus template plus enzyme, , primer plus template plus enzyme plus 4 µM dNTPαS controls were also carried out. The reactions were incubated at 37°C for 3 minutes **(4 and 5)** or 10 minutes **(6)**. Reactions were then stopped by the addition of formamide / EDTA stop solution. Reaction products were separated on a 19% polyacrylamide 7M urea gel. After exposure to Kodak Biomax autoradiography film the incorporation of the analogue was studied by comparison to the control reactions using either primer alone or primer plus template plus enzyme and 4 µM dNTPαS.

This showed that compounds **(4, 5 and 6)** were good substrates for EFK and that each was incorporated in place of dTTP against Template 1 above. No incorporation in place of dCTP was seen on either template.

CLAIMS

1. A compound having the structure



where X is CH or N,

Y is Y is -CO-, -CONW-, -O-, -S-, -SO-, -SO_2-, -NWCO-,-NW-, or -OCO-,

W is the same or different at different places in the molecule and each is H or alkyl or aryl or Rp or –Ln–Rp,

Ln is a linker group,

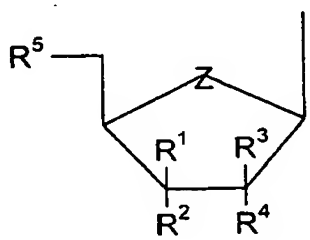
Rp is a reporter moiety, and

Q is H or a sugar or a sugar analogue or a nucleic acid backbone or backbone analogue,

provided that at least one reporter moiety Rp is present.

2. The compound as claimed in claim 1, which is

a) a nucleoside or nucleoside analogue wherein Q is



where Z is O, S, Se, SO, NW or CH_2,

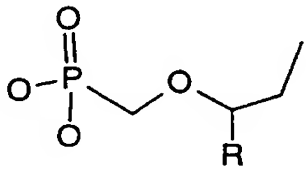
R^1, R^2, R^3 and R^4 are the same or different and each is H, OH, F, NH_2, N_3, O-hydrocarbyl or Rp or –Ln–Rp,

R^5 is OH, SH or NH_2 or mono-, di- or tri-phosphate or -thiophosphate, or corresponding boranophosphate,

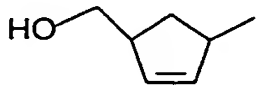
~~or one of R^2 and R^5 is a phosphoramidite or other group for~~ incorporation in a polynucleotide chain, or a reporter moiety,

or Q consists of one of the following modified sugar structures

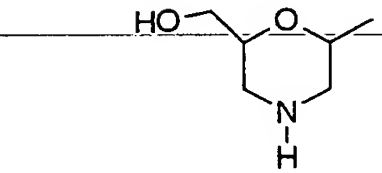
Acyclic Sugars

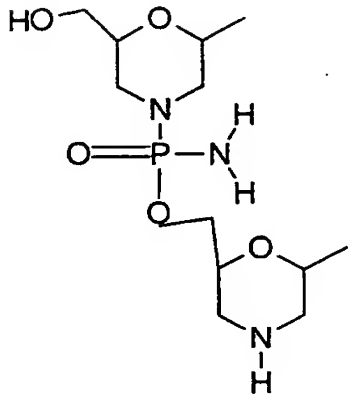


R= CH_3 , CH_2OH , H ,



Morpholino Backbone





or b) a polynucleotide wherein Q is a nucleic acid backbone consisting of sugar-phosphate repeats or modified sugar-phosphate repeats (LNA), or a backbone analogue such as peptide or polyamide nucleic acid (PNA).

3. The compound of claim 1 or claim 2, wherein a reporter moiety Rp is not present in Q.

4. The compound of any one of claims 1 to 4, which is a nucleoside analogue.

5. The compound of any one of claims 1 to 4, wherein the linker group Ln is a chain of 1 to 60 carbon, nitrogen, oxygen, phosphorus and/or sulphur atoms, rigid or flexible, saturated or unsaturated.

6. The compound of any one of claims 1 to 5, wherein the reporter moiety Rp is a signal moiety or a solid surface or a reactive group by means of which a signal moiety or a solid surface may be linked to the nucleoside analogue.

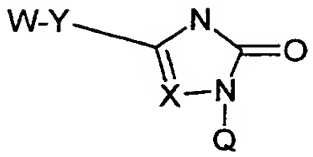
7. The compound of claim 6, wherein the reactive group is NH_2, OH, COOH, $CONH_2$, ONH_2, SH, or a thiophosphate or a hydrazine or a hydrazide, or an active ester or aldehyde or maleimide.

8. The nucleotide analogue of any one of claims 4 to 7, wherein R^5 is triphosphate.

9. A polynucleotide chain comprising at least one residue of the nucleoside analogue of any one of claims 4 to 8.

ABSTRACT

Compounds having the structure



where X is CH or N,

Y is Y is -CO-, -CONW-, -O-, -S-, -SO-, -SO_2-, -NWCO-,-NW-, or -OCO-,

W is the same or different at different places in the molecule and each is H or alkyl or aryl or Rp or –Ln–Rp,

Ln is a linker group,

Rp is a reporter moiety, and

Q is H or a sugar or a sugar analogue or a nucleic acid backbone or backbone analogue,

provided that at least one reporter moiety Rp is present, provide nucleoside triphosphates which are good enzyme substrates.